and *Function of Metalloproteins* (Darnall, D. W., & Wilkins, R. G., Eds.) pp 27–74, Elsevier/North-Holland, New York.

Vallee, B. L., Riordan, J. F., & Coleman, J. E. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 109–116.

Vallee, B. L., Galdes, A., Auld, D. S., & Riordan, J. F. (1983) in *Metal Ions in Biology* (Spiro, T. G., Ed.) Vol. 5, Wiley, New York (in press).

Van Wart, H. E., & Vallee, B. L. (1978) *Biochemistry 17*, 3385–3394.

# Kinetic and Electrophoretic Properties of Native and Recombined Isoenzymes of Human Liver Alcohol Dehydrogenase[†]

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ABSTRACT: Ten, electrophoretically distinct, molecular forms of alcohol dehydrogenase have been isolated from a single human liver by affinity and ion-exchange chromatography. The starch gel electrophoresis patterns after the dissociation–recombination of the forms are consistent with the hypothesis that they arise from the random combination of $\alpha$, $\beta_1$, $\gamma_1$, and $\gamma_2$ subunits into six heterodimeric and four homodimeric isoenzymes. Large differences in kinetic properties are observed for the homodimeric isoenzymes, $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$. At pH 7.5, the $K_m$ value of $\beta_1\beta_1$ for ethanol is 0.049 mM and that of $\alpha\alpha$ is 4.2 mM. Forms $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ do not obey Michaelis–Menten kinetics at pH 7.5 but exhibit negative cooperativity with Hill coefficients of 0.54 and 0.55 and $[S]_{0.5}$ values of 1.0 and 0.63 mM, respectively. However, all isoenzymes display Michaelis–Menten kinetics for ethanol oxidation at pH 10.0 with $K_m$ values ranging from 1.5 to 3.2 mM. The maximum specific activity of $\beta_1\beta_1$ is considerably lower than that of the other three homodimers at both pH 7.5 and 10.0. The $K_m$ values of the four homodimers for $NAD^+$ at pH 7.5 range from 7.4 to 13 $\mu$M and those for NADH, from 6.4 to 33 $\mu$M. $K_i$ values for NADH range from 0.19 to 1.6 $\mu$M. At pH 7.5, the kinetic properties of $\alpha\alpha$ and $\beta_1\beta_1$, prepared in vitro from dissociated and recombined $\alpha\beta_1$, are similar to those of the native homodimers. The forms $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$, prepared from dissociated and recombined $\alpha\gamma_1$ and $\beta_1\gamma_2$, respectively, exhibit negative cooperativity with Hill coefficients that are similar to those seen with the respective native homodimers.

Human liver alcohol dehydrogenase (EC 1.1.1.1), a dimeric enzyme, exhibits multiple, electrophoretically distinct, molecular forms (Smith et al., 1971; Schenker et al., 1971; Pietruszko et al., 1972; Harada et al., 1978; Bosron & Li, 1981) which can be divided into three classes according to their functional and structural properties (Vallee & Bazzone, 1983). Those isoenzymes formed by the random combination of subunits $\alpha$, $\beta$, and $\gamma$, named according to the scheme of Smith et al. (1971), belong to class I. They migrate as a group toward the cathode on starch gel electrophoresis at pH 7.7–8.6 and are inhibited by micromolar concentrations of 4-methylpyrazole. The tryptic peptide elution profiles of these enzyme forms obtained with high-performance liquid chromatography are similar to one another, suggesting close structural homology within the group (Strydom & Vallee, 1982). The enzyme forms in class II, $\pi$-ADH, and class III, $\chi$-ADH, differ substantially from class I isoenzymes with respect to electrophoretic mobility on starch gels, sensitivity to inhibition by 4-methylpyrazole, $K_m$ values for ethanol, and tryptic peptide elution profiles (Vallee & Bazzone, 1982).

A system of nomenclature for the class I molecular forms was proposed some time ago, based on an isoenzyme model involving three structural genes.[1] It was postulated that a single subunit, $\alpha$, is produced at the $ADH_1$ gene locus, two allomorphic subunits, $\beta_1$ and $\beta_2$, are produced at $ADH_2$, and two allomorphic subunits, $\gamma_1$ and $\gamma_2$, are produced at $ADH_3$ (Smith et al., 1971). These subunits would combine randomly to form hetero- and homodimers. Evidence in support of this hypothesis was obtained in studies of enzyme electrophoretic patterns following the dissociation–recombination of purified isoenzyme mixtures (Smith et al., 1973). For example, $\alpha\alpha$ and $\beta_1\beta_1$ were obtained from fetal liver and from lung, tissues that preferentially express these forms, and upon dissociation–recombination, a new heterodimer with intermediate electrophoretic mobility, $\alpha\beta_1$, was generated. However, because convenient and effective methods for the complete purification of alcohol dehydrogenase isoenzymes from human liver became available only recently, a systematic and more definitive study using the entire set of enzyme forms purified from the same liver had yet to be performed.

We report here the purification from a single liver of the ten class I alcohol dehydrogenase molecular forms postulated to arise from the combination of $\alpha$, $\beta_1$, $\gamma_1$, and $\gamma_2$ subunits and the results of the dissociation–recombination of each of these enzyme forms. To substantiate that the subunits in the heterodimers are identical with those present in homodimers, we have isolated the homodimers produced in vitro from dissociated and recombined heterodimers and compared their kinetic properties with those of the native homodimers. Although prior studies of several partially purified isoenzymes of uncertain identity revealed only minor differences in substrate specificity or kinetic properties (Pietruszko et al., 1972), the

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[1] Gene loci and phenotypes are named according to Giblett (1976). Alcohol dehydrogenase loci are designated by a subscript, e.g., $ADH_3$. Polymorphic alleles are designated by a superscript above the gene locus, e.g., $ADH_3^1$. Phenotypes are identified by the subscript for the gene locus followed by the designation of the 2 alleles present on each copy of the gene. Hence, for the $ADH_3$ 2-1 phenotype, both the $ADH_3^2$ and $ADH_3^1$ alleles are present, and the individual is heterozygous at the $ADH_3$ locus.

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studies reported here on the four purified homodimeric isoenzymes demonstrate that they differ substantially in ethanol saturation profile, $K_m$ or $[S]_{0.5}$ values for ethanol and acetaldehyde, and maximum specific enzymatic activity (Bosron & Li, 1982; Bosron et al., 1982).

## Experimental Procedures

Alcohol dehydrogenase activity in liver homogenate supernatants was assayed spectrophotometrically at 340 nm with 33 mM ethanol and 2.4 mM $NAD^+$ (grade I; Boehringer-Mannheim, Indianapolis, IN) in 0.1 M glycine–NaOH at pH 10.0, 25 °C. Enzyme activity units are expressed as micromoles of NADH formed or utilized per minute based on an $A_{340nm}$ of 6.22 $mM^{-1}$ $cm^{-1}$. Alcohol dehydrogenase isoenzymes were identified by high-voltage starch gel electrophoresis at pH 8.2 (Bosron et al., 1979b). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with an LKB Multiphor apparatus (LKB Instruments, Rockville, MD), and protein was stained with Coomassie Blue. All chemicals were reagent grade, and water was deionized and distilled.

Human liver specimens were obtained at autopsy within 12 h of death from apparently healthy individuals who had succumbed to sudden, traumatic death. Information on race, age, sex, and principal cause of death was obtained from autopsy records with assurance to protect the anonymity of the donors. Liver specimens were stored at −55 °C. Alcohol dehydrogenase isoenzymes were isolated by affinity and ion-exchange chromatography. Livers were homogenized in 2 volumes of distilled water, and homogenates were centrifuged at 69000*g* for 60 min. The homogenate–supernatant was passed through a column containing 5 mL of packed DEAE-cellulose/g of liver (DE-52, Whatman, Clifton, NJ), equilibrated with 10 mM Tris-HCl[2] at pH 8.0. Tris-HCl buffer, pH 8.0, and $NAD^+$ were added to the effluent to a concentration of 100 and 3 mM, respectively, and the solution was applied to a CapGapp–Sepharose[2] affinity column (Lange & Vallee, 1976). Approximately 10–30% of the enzyme activity did not bind to the resin and eluted in the void volume with the majority of the protein. This fraction contained $\pi$-alcohol dehydrogenase (Li et al., 1977). The enzyme forms that bound to CapGapp–Sepharose were eluted with 0.5 M ethanol in 100 mM Tris-HCl, pH 8.0. The recovery of activity in this purification step was 80–95%.

Separation of isoenzymes was accomplished by ion-exchange chromatography on CM-cellulose (CM-52, Whatman, Clifton, NJ) in 5 mM Tris–$P_i$ and 1.0 mM $NAD^+$, pH 7.7 at 4 °C, with a linear gradient of 0–40 mM NaCl in buffer (Figure 1). The recovery of activity was 75–85%. Those fractions containing single isoenzymes were precipitated with $(NH_4)_2SO_4$, 0.6 g/mL, and stored at −55 °C. Mixtures of isoenzymes were further purified by chromatography on CM-cellulose equilibrated in 12.5 mM $NaP_i$ and 10 mM ethanol, pH 6.7 at 4 °C, and eluted with a 12.5–40 mM linear gradient of buffer (Figure 2). All molecular forms were gel filtered on Bio-Gel P-6 (Bio-Rad, Richmond, CA) in 5 mM $NaP_i$, pH 7.5, before further experiments were performed.

Dissociation of the isolated isoenzymes into subunits and recombination of them into catalytically active dimers were performed by a modification of the freeze–thaw method described by Hart (1971). Enzyme was frozen for 18 h at −55 °C in 0.1 M $NaP_i$, 1.0 M NaCl, 0.4 M sucrose, and 0.1 M 2-mercaptoethanol, pH 7.5. The samples were rapidly thawed and dialyzed for 24 h at 4 °C against two changes of 10 mM $NaP_i$, 0.57 mM $NAD^+$ (grade AA; Sigma Chemical Co., St. Louis, MO) and 50 μM $ZnSO_4$, pH 7.0. Recovery of activity ranged from 40 to 80%.

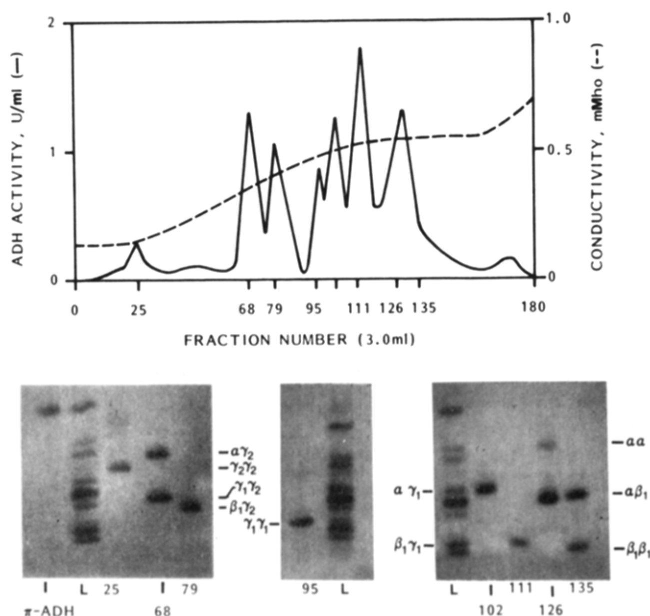


FIGURE 1: Separation and electrophoresis of ADH isoenzymes from a liver with the $ADH_2$ 1-1, $ADH_3$ 2-1 phenotype. The 10 isoenzymes that bound to CapGapp–Sepharose were eluted with ethanol. The upper panel shows the activity elution profile for the separation of these 10 enzyme forms by chromatography on CM-cellulose at pH 7.7. The lower panels compare the starch gel electrophoresis patterns of the seven major activity peaks eluted from CM-cellulose with the isoenzymes present in the homogenate supernatant (lane L). $\pi$-ADH was recovered in the CapGapp–Sepharose void volume. The gels were stained for ethanol oxidizing activity.

Steady-state kinetic studies were performed in 0.1 M $NaP_i$, pH 7.5 at 25 °C, by measuring the production or utilization of NADH in the presence of ethanol or acetaldehyde with a Varian 219 spectrophotometer or an Aminco-Bowman J4-8203 H spectrophotofluorometer. The fluorometer was calibrated with NADH solutions by excitation at 340 nm and emission at 455 nm. All kinetic data were obtained in duplicate or triplicate, and steady-state kinetic constants were evaluated by the statistical programs of Cleland (1979). The coefficient of variation for $K_m$ and $V_{max}$ with all substrates was 12% or less. That for the substrate inhibition constant of $\alpha\alpha$ and $\beta_1\beta_1$ was 8–26%, because $K_i$ exceeded the highest ethanol concentration employed, 250 mM. The concentration of enzyme active sites was determined by fluorescence titration of the binary enzyme–isobutyramide complex with NADH (Pietruszko et al., 1972).

## Results

*Isolation and Dissociation–Recombination of Alcohol Dehydrogenase Class I Isoenzymes Containing $\alpha$, $\beta_1$, $\gamma_1$, and $\gamma_2$ Subunits.* Ten class I isoenzymes were purified from a liver exhibiting the electrophoresis pattern shown in Figure 1 (lane L) by double-ternary complex chromatography on CapGapp–Sepharose (Lange & Vallee, 1976). The class II enzyme form, $\pi$-ADH, was recovered in the void fraction from the CapGapp–Sepharose column (Li et al., 1977). The 10 class I forms were separated into seven peaks of enzyme activity by ion-exchange chromatography on CM-cellulose at pH 7.7 (Figure 1). Five peaks contained single isoenzymes as shown by starch gel electrophoresis of fractions 25, 79, 95, 102, and 111 in Figure 1. The other peaks, fractions 68, 126, and 135, each contained two or three isoenzymes. The iso-

[2] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; CapGapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole.

Table I: Kinetic Constants of Native Homodimers for Ethanol Oxidation at pH 7.5

| | isoenzyme | | | |
|---|---|---|---|---|
| | $\alpha\alpha$ | $\beta_1\beta_1$ | $\gamma_1\gamma_1$ | $\gamma_2\gamma_2$ |
| $K_m$, ethanol (mM)[a] | 4.2 ± 0.9 | 0.049 ± 0.018 | | |
| $K_i$, ethanol (mM)[a] | 830 ± 190 | 440 ± 240 | b | b |
| Hill coefficient[a,c] | 0.98 ± 0.03 | 0.94 ± 0.17 | 0.54 ± 0.14 | 0.55 ± 0.03 |
| $[S]_{0.5}$ (mM)[c] | | | 1.0 ± 0.3 | 0.63 ± 0.20 |
| $V_{max}$ (units/µmol of active site)[a,c] | 27 ± 4 | 9.2 ± 0.9 | 87 ± 7 | 35 ± 8 |
| $K_m$, NAD$^+$ (µM)[d] | 13 | 7.4 | 7.9 | 8.7 |

[a] Values are the mean ± SD for three preparations of $\alpha\alpha$ from different livers and for six preparations of $\beta_1\beta_1$. NAD$^+$ concentration was 2.4 mM. [b] Ethanol substrate inhibition was not observed. [c] Values are the mean ± SD for five preparations of $\gamma_1\gamma_1$ and three of $\gamma_2\gamma_2$. NAD$^+$ concentration was 2.4 mM. [d] Values are the mean for two isoenzyme preparations. Ethanol concentration was 50 mM for $\alpha\alpha$, 5 mM for $\beta_1\beta_1$, and 100 mM for $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$.

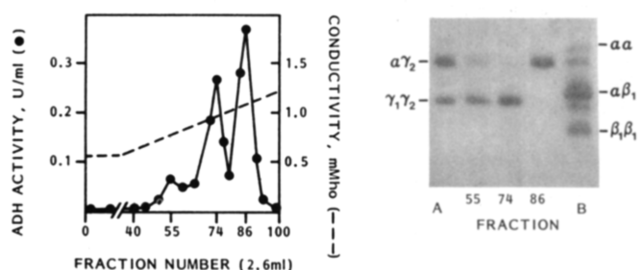


FIGURE 2: Separation and electrophoresis of the $\alpha\gamma_2$ and $\gamma_1\gamma_2$ isoenzymes. The electrophoresis pattern of the isoenzymes in peak fraction 68, Figure 1, is shown in lane A. The elution profile of activity after chromatography of this mixture on CM-cellulose at pH 6.7 is shown on the left. On the right, the electrophoresis pattern of enzyme in fractions 55, 74, and 86 is compared with that in a liver homogenate supernatant exhibiting the ADH$_2$ 1-1, ADH$_3$ 2-2 phenotype (lane B). The gel was stained for ethanol oxidizing activity.

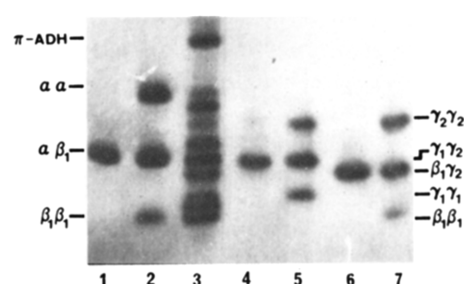


FIGURE 3: Starch gel electrophoresis of $\alpha\beta_1$, $\gamma_1\gamma_2$, and $\beta_1\gamma_2$ before and after dissociation–recombination treatment. The electrophoresis patterns of purified $\alpha\beta_1$, $\gamma_1\gamma_2$, and $\beta_1\gamma_2$ are shown in lanes 1, 4, and 6, respectively, and their patterns after dissociation–recombination treatment are shown in lanes 2, 5, and 7, respectively. The electrophoresis pattern of the original liver homogenate–supernatant with the ADH$_2$ 1-1, ADH$_3$ 2-1 phenotype is shown in lane 3. The gel was stained for ethanol oxidizing activity.

enzymes in each of these peaks were separated from one another by rechromatography on CM-cellulose at pH 6.7. An example of the separation of the two isoenzymes in peak fraction 68 is shown in Figure 2. All purified isoenzymes exhibited a single band of 40 000 daltons on sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The electrophoresis patterns generated by the dissociation–recombination of each of the 10 purified class I isoenzymes were examined. Four of the purified isoenzymes remained as single bands after the freeze–thaw treatment, and their electrophoretic mobility was identical with that of the original undissociated isoenzyme; therefore, they are homodimers. On the basis of the nomenclature of Smith et al. (1971), these four homodimers are identified as $\gamma_2\gamma_2$, $\gamma_1\gamma_1$, $\alpha\alpha$, and $\beta_1\beta_1$ (Figure 1). The other six isoenzymes are heterodimers since three bands appeared after dissociation–recombination. The electrophoretic mobilities of the two new bands formed after treatment were compared with those of the four homodimeric isoenzymes described above. The isoenzyme in lane 1 of Figure 3 is $\alpha\beta_1$, since $\alpha\alpha$ and $\beta_1\beta_1$ were generated after dissociation–recombination (lane 2, Figure 3). In similar fashion, the remaining five heterodimers are identified as $\gamma_1\gamma_2$ (lanes 4 and 5, Figure 3), $\beta_1\gamma_2$ (lanes 6 and 7, Figure 3), and $\alpha\gamma_1$, $\alpha\gamma_2$, and $\beta_1\gamma_1$ (electrophoresis patterns not shown).

The form $\pi$-ADH was purified from the CapGapp–Sepharose void fractions by affinity chromatography on agarose–hexane–AMP (Li et al., 1977). Dissociation–recombination treatment of $\pi$-ADH together with either $\alpha\alpha$, $\gamma_1\gamma_1$, $\gamma_2\gamma_2$, or $\beta_1\beta_1$ did not generate any new electrophoretic bands, suggesting that subunits from these isoenzymes cannot recombine with subunits from $\pi$-ADH to form heterodimers.

*Steady-State Kinetic Properties of the Four Native Homodimeric Alcohol Dehydrogenase Isoenzymes.* The kinetic constants for purified $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ were determined

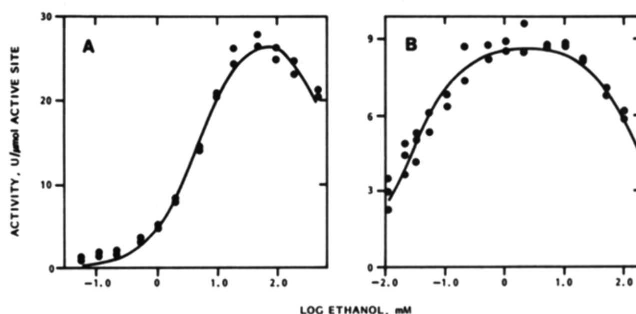


FIGURE 4: Ethanol saturation profiles of $\alpha\alpha$ and $\beta_1\beta_1$. Enzyme activity was determined in 0.1 M NaP$_i$, pH 7.5 at 25 °C, with 2.4 mM NAD$^+$. The line is the fit of data to eq 1. $K_m$, $K_i$, and $V_{max}$ for $\alpha\alpha$, panel A, are 5.3 ± 0.4 mM, 920 ± 120 mM, and 30 ± 1 units/µmol of active site; for $\beta_1\beta_1$, panel B, they are 0.025 ± 0.002 mM, 200 ± 20 mM, and 8.8 ● 0.1 units/µmol of active site, respectively.

in 0.1 M NaP$_i$ at pH 7.5 and 25 °C. The substrate saturation curves for $\alpha\alpha$ (panel A, Figure 4) and $\beta_1\beta_1$ (panel B, Figure 4) fit the second-order polynomial expression for substrate inhibition (Cleland, 1979):

$$v/V_{max} = S/[K_m + S + (S^2/K_i)] \quad (1)$$

In this equation, $v$ is the initial velocity, $V_{max}$ is the maximal velocity, $S$ is the ethanol concentration, $K_m$ is the Michaelis constant for ethanol, and $K_i$ is the substrate inhibition constant for ethanol. The average values and standard deviations of $K_m$, $K_i$, and $V_{max}$ of native $\alpha\alpha$ and $\beta_1\beta_1$ prepared from several different livers, calculated from eq 1, are shown in Table I.

The homodimers $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ did not obey simple Michaelis–Menten kinetics over a wide range of ethanol concentrations (0.05–100 mM) at pH 7.5. However, as shown in Figure 5, after $V_{max}$ values were estimated from the fit of velocities obtained at high ethanol concentrations (10–100

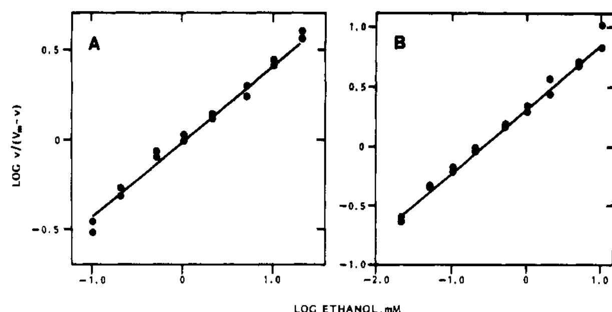


FIGURE 5: Hill plots for $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$. The line is the linear regression of data to eq 2. The regression coefficients are 0.99 for both $\gamma_1\gamma_1$ (panel A) and $\gamma_2\gamma_2$ (panel B). The hill coefficient ($h$) is 0.42 for $\gamma_1\gamma_1$ and 0.53 for $\gamma_2\gamma_2$, and their $[S]_{0.5}$ values are 1.0 and 0.49, respectively. $V_{max}$ for $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ is 94 and 32 units/$\mu$mol of active site, respectively, calculated by fitting enzyme velocities at high ethanol concentration (10–100 mM) to the Michaelis–Menten equation (Cleland, 1979).

Table II: Kinetic Constants of Native Homodimers for Acetaldehyde Reduction and NADH Product Inhibition at pH 7.5

| | isoenzyme | | | |
|---|---|---|---|---|
| | $\alpha\alpha$ | $\beta_1\beta_1$ | $\gamma_1\gamma_1$ | $\gamma_2\gamma_2$ |
| $K_m$, acetaldehyde (mM)[a] | 4.3 | 0.085 | 0.33 | 0.24 |
| $K_m$, NADH ($\mu$M)[b] | 11 | 6.4 | 7.0 | 33 |
| $K_i$, NADH ($\mu$M)[c] | 0.40 | 0.19 | 0.98 | 1.6 |
| $V_{max}$ (units/$\mu$mol of active site)[a] | 700 | 240 | 2100 | 1400 |
| $V_{max}(K_i/K_m)$ (NADH)[d] | 25 | 7.1 | 290 | 68 |

[a] Values are the mean for two isoenzyme preparations. NADH concentration was 0.2 mM. Substrate inhibition was observed above 30 mM acetaldehyde for $\beta_1\beta_1$ but not for $\alpha\alpha$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$. [b] Acetaldehyde concentration was 40 mM for $\alpha\alpha$, 30 mM for $\beta_1\beta_1$, and 10 mM for $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$. [c] Ethanol concentration was 50 mM for $\alpha\alpha$, 5 mM for $\beta_1\beta_1$, and 100 mM for $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$. The $NAD^+$ concentration ranged from 7 to 70 $\mu$M for all homodimers. [d] This product equals the rate constant for dissociation of NADH from E-NADH for the ordered sequential mechanism (Cleland, 1963).

mM) to the Michaelis–Menten equation (Cleland, 1979), the data could be fit to a kinetic equation for cooperativity, the Hill equation (Whitehead, 1978):

$$\log \frac{v}{V_{max} - v} = h \log S - h \log [S]_{0.5} \qquad (2)$$

The average values of the Hill coefficient ($h$) for $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ prepared from several livers were 0.54 and 0.55, respectively, indicating negative cooperativity for ethanol saturation, and the average $[S]_{0.5}$ values for ethanol were 1.00 and 0.63 mM, respectively (Table I). As expected, Hill coefficients for ethanol with $\alpha\alpha$ and $\beta_1\beta_1$ were nearly 1.0 (Table I).

All four homodimers exhibited linear double-reciprocal plots when acetaldehyde, $NAD^+$, or NADH concentration was varied and the respective cosubstrate was fixed at a saturating concentration; their average $K_m$ values from duplicate determinations are listed in Tables I and II. All four homodimers exhibited a competitive inhibition pattern when $NAD^+$ concentration was varied at a saturating ethanol concentration and NADH was the inhibitor; their average values for the slope inhibition constant, $K_i$, are listed in Table II. Linear intersecting double-reciprocal plots were obtained for $\alpha\alpha$ and $\beta_1\beta_1$ when $NAD^+$ and ethanol concentrations were covaried and ethanol was a mixed noncompetitive inhibitor of acetaldehyde at saturating NADH concentrations. These inhibition patterns of $\alpha\alpha$ and $\beta_1\beta_1$ are similar to data previously reported for $\beta_1\beta_1$ (Dubied et al., 1977), horse liver alcohol dehydrogenase (Theorell & Chance, 1951; Wratten & Cleland, 1963), and

Table III: Kinetic Constants of Native Homodimers for Alcohol Oxidation at pH 10.0[a]

| | isoenzyme | | | |
|---|---|---|---|---|
| | $\alpha\alpha$ | $\beta_1\beta_1$ | $\gamma_1\gamma_1$ | $\gamma_2\gamma_2$ |
| $K_m$, ethanol (mM) | 1.5 | 1.6 | 3.2 | 1.7 |
| $K_i$, ethanol (mM) | 120 | 360 | *b* | *b* |
| $V_{max}$ (units/$\mu$mol of active site) | 150 | 18 | 220 | 120 |

[a] Values are the mean for two isoenzyme preparations. $NAD^+$ concentration was 2.4 mM. [b] Substrate inhibition was not observed.

human liver $\pi$-alcohol dehydrogenase (Bosron et al., 1979a); they are consistent with an ordered sequential mechanism.

In agreement with the observations of Burger & Vallee (1981), the ethanol saturation kinetics of $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ at pH 10.0 fit the equations for simple Michaelis–Menten kinetics ($\gamma_1\gamma_1$ and $\gamma_2\gamma_2$) or substrate inhibition ($\alpha\alpha$ and $\beta_1\beta_1$). $K_m$ values of the four homodimers ranged from 1.5 to 3.2 mM (Table III). The $V_{max}$ values of $\beta_1\beta_1$ for ethanol at both pH 10.0 and pH 7.5 were considerably lower than those of the other three homodimers (Tables I and III).

*Isolation and Steady-State Kinetics of Homodimers Prepared by Dissociation–Recombination of Heterodimers.* Native $\alpha\beta_1$ was dissociated and recombined by the freeze–thaw procedure (Figure 3, lane 2), and the newly generated $\alpha\alpha$ and $\beta_1\beta_1$ were isolated by CM-cellulose chromatography at pH 6.7. In a similar manner, $\gamma_1\gamma_1$ and $\alpha\alpha$ were isolated after dissociation–recombination of $\alpha\gamma_1$, and $\gamma_2\gamma_2$ and $\beta_1\beta_1$ were isolated after dissociation–recombination of $\beta_1\gamma_2$ (Figure 3, lane 7). At pH 7.5, $\alpha\alpha$ and $\beta_1\beta_1$ exhibited ethanol saturation curves that were similar to those of the native homodimers, and their $K_m$ values were 3.9 and 0.052 mM, respectively. Hill coefficients for recombined $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ were 0.56 and 0.68, respectively, again indicating negative cooperativity for ethanol saturation, and $[S]_{0.5}$ values were also similar to those of the native homodimers.

## Discussion

Rapid and efficient affinity and ion-exchange chromatography methods have been developed recently for the purification of alcohol dehydrogenases (Lange & Vallee, 1976; Li et al., 1977; Bosron et al., 1980), and these methods were employed for the purification of all the class I isoenzymes from a human liver with the phenotype shown in Figure 1. We have adopted the scheme for naming these enzyme forms that is based on the electrophoretic mobilities of dimeric isoenzymes whose subunits are presumed to be products of three different structural genes (Smith et al., 1971). The phenotype for the liver shown in Figure 1 is $ADH_2$ 1-1, $ADH_3$ 2-1, because it contains isoenzymes with $\alpha$, $\beta_1$, $\gamma_1$, and $\gamma_2$ subunits. The $\alpha$ and $\beta_1$ subunits are products of $ADH_1$ and $ADH_2$, respectively, and the $\gamma_1$ and $\gamma_2$ subunits are products of the polymorphic $ADH_3$ locus. According to the scheme, four homodimers, $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$, and six heterodimers, $\alpha\beta_1$, $\alpha\gamma_1$, $\alpha\gamma_2$, $\beta_1\gamma_1$, $\beta_1\gamma_2$, and $\gamma_1\gamma_2$, should be formed by the random combination of the four subunits. We have now verified this assumption by purifying all 10 isoenzymes from a single liver (Figures 1 and 2) and examining their electrophoretic patterns after dissociation–recombination (Figure 3). Comparison of the kinetic properties of the four homodimers produced in vitro by the dissociation–recombination of heterodimers with the kinetic properties of the native homodimers confirms that the $\alpha$, $\beta_1$, $\gamma_1$, and $\gamma_2$ subunits in the homo- and heterodimers are identical.

Freeze–thaw treatment of these four homodimers together with π-ADH, purified from the same liver (Figure 1), indicates that the subunits of π-ADH do not recombine with those from the class I homodimers. It has been reported that anti-$\beta_1\beta_1$ antibody does not cross-react with π-ADH (Adinolfi et al., 1978) and that the tryptic peptide elution profiles for π-ADH differ substantially from those of isoenzymes composed of α, β, or γ subunits (Strydom & Vallee, 1982). All these observations point to the existence of substantial structural differences between these two classes of alcohol dehydrogenases.

The ethanol saturation profiles for αα and $\beta_1\beta_1$ at pH 7.5 obey simple substrate inhibition kinetics and product inhibition patterns are consistent with the ordered sequential mechanism that was proposed some years ago for horse liver alcohol dehydrogenase (Theorell & Chance, 1951; Wratten & Cleland, 1963). According to the Theorell–Chance mechanism, the $V_{max}$ for ethanol oxidation is determined by the rate of dissociation of NADH from the binary E–NADH complex, and this rate constant is equal to the $V_{max}$ for acetaldehyde reduction times the ratio $K_i/K_m$ for NADH (Cleland, 1963). The rate constants of αα and $\beta_1\beta_1$ for NADH dissociation, calculated from the kinetic constants in Table II, agree well with the $V_{max}$ values for ethanol oxidation (Table I). Therefore, αα and $\beta_1\beta_1$ obey the rate-limiting condition of the Theorell–Chance mechanism.

By contrast, $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ at pH 7.5 do not obey the Theorell–Chance mechanism, since they display negative cooperativity for ethanol saturation (Figure 5), and the product of $V_{max}$ for aldehyde reduction and $K_i/K_m$ for NADH (Table II) does not agree with the $V_{max}$ for ethanol oxidation (Table I). Further, since both $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ exhibit Michaelis–Menten kinetics when $NAD^+$ concentration is varied, the negative cooperativity cannot be explained by the random order of addition of $NAD^+$ and ethanol to enzyme (Wratten & Cleland, 1963). Such behavior can be due to the presence of two or more enzyme forms in these preparations, to half-site reactivity, or to a yet unidentified mechanism. However, multiple enzyme forms were not detected in these preparations by high-voltage starch gel electrophoresis, and both $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ prepared in vitro from purified heterodimers also displayed negative cooperativity. Moreover, because αα and $\beta_1\beta_1$ exhibit substrate inhibition above 50 mM ethanol at pH 7.5 but $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ do not display this property, preparations of $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ are not likely to be contaminated with isoenzymes containing α or $\beta_1$ subunits. Interestingly, both $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ also obey simple Michaelis–Menten kinetics for acetaldehyde reduction at pH 7.5 and 10.0, and for ethanol oxidation at pH 10.0. Thus, negative cooperativity pertains only to ethanol at pH 7.5. Studies on the rate and equilibrium of binding of substrates and products to these forms will need to be performed as a function of pH to identify the underlying mechanism of this kinetic behavior.

Initial studies of the kinetic properties of several partially purified human liver alcohol dehydrogenase isoenzymes had detected only minor differences in their $K_m$ values for ethanol at pH 7.0 and 10.0 (Pietruszko et al., 1972). The studies here reported indicate that this is clearly not so. While the $K_m$ values of all four homodimers are similar at pH 10.0 (Table III; Burger & Vallee, 1981), $\beta_1\beta_1$ exhibits a $K_m$ value that is 100 times lower than that for αα and a value 13–20 times lower than the $[S]_{0.5}$ values of $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ at pH 7.5 (Table I). Moreover, the maximum ethanol oxidizing activity ($V_{max}$) of $\beta_1\beta_1$ is 3–9 times lower than those of the three other homodimers at pH 7.5 (Table I) and 7–12 times lower than those at pH 10.0 (Table III). The $K_m$ values of the four homodimers for $NAD^+$ at pH 7.5, however, are similar (Table II). Substantial differences were also observed among the four homodimers in the $V_{max}$ for acetaldehyde reduction at pH 7.5 and the $K_m$ values for acetaldehyde and NADH (Table II).

The $K_m$ of $\beta_1\beta_1$ for ethanol reported here differs substantially from that reported by Bühler & von Wartburg (1982) at pH 7.0. The reason for this discrepancy is not apparent. However, because the $V_{max}$ values of $\beta_1\beta_1$ in both the ethanol oxidizing and the acetaldehyde reducing directions are substantially lower than those of the other three homodimers, even a small degree of contamination of $\beta_1\beta_1$ with isoenzymes containing α, $\gamma_1$, or $\gamma_2$ subunits would substantially alter the kinetic analysis of $\beta_1\beta_1$. Therefore, it is important that methods such as high-voltage starch gel electrophoresis (Harada et al., 1978; Bosron et al., 1980), isoelectric focusing,[3] or peptide analysis (Yoshida et al., 1981; Strydom & Vallee, 1982) be used to assess purity of the preparations when the kinetics of $\beta_1\beta_1$ are studied.

As shown in Tables I and III, the effect of pH on the kinetics of ethanol oxidation differs substantially among the four homodimers. The $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ isoenzymes exhibit negative cooperativity for ethanol oxidation at pH 7.5, but they obey simple Michaelis–Menten kinetics at pH 10.0. The αα and $\beta_1\beta_1$ isoenzymes obey simple substrate inhibition kinetics at both pH 7.5 and 10.0; however, the $K_m$ of $\beta_1\beta_1$ for ethanol *increases* 33-fold when the pH is increased from 7.5 to 10.0, whereas that of αα *decreases* 64% under these conditions (Tables I and III). On the other hand, all four homodimers exhibit similar pH–activity profiles with a maximum value at pH 10–10.5 when measured at 25 °C with 2.4 mM $NAD^+$ and 33 mM ethanol[3] (Bosron et al., 1980). Since these concentrations of substrates are saturating for all four homodimers at both pH 7.5 and pH 10.0 (Tables I and III), the pH dependence of activity must be due to a change in $V_{max}$, and this is confirmed by comparing $V_{max}$ values at pH 7.5 (Table I) with those at 10.0 (Table III). In this regard, it is of interest that there are also variant homodimeric forms of human liver alcohol dehydrogenase that exhibit pH optima for ethanol oxidation at 8.5 and at 7.0. It is presumed that these forms are alloenzymes of $\beta_1\beta_1$ produced by the $ADH_2$ gene locus and have been designated $\beta_2\beta_2$ and $\beta_{Ind}\beta_{Ind}$, respectively (Yoshida et al., 1981; Bosron et al., 1980). An examination of the kinetics of ethanol oxidation by these variant enzymes as a function of pH should reveal whether the difference in pH optima among the forms is due to a pH-dependent change in $V_{max}$ or in $K_m$ for ethanol or $NAD^+$ or to a combination of these alterations.

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**Registry No.** NAD, 53-84-9; NADH, 58-68-4; ethanol, 64-17-5; acetaldehyde, 75-07-0; alcohol dehydrogenase, 9031-72-5.

References

Adinolfi, A., Adinolfi, M., Hopkinson, D. A., & Harris, H. (1978) *J. Immunogenet. 5*, 283–296.

Bosron, W. F., & Li, T.-K. (1981) *Semin. Liver Dis. 1*, 179–188.

[3] S.-J. Yin, W. F. Bosron, and T.-K. Li, unpublished observations.

Bosron, W. F., & Li, T.-K. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 921.

Bosron, W. F., Li, T.-K., Dafeldecker, W. P., & Vallee, B. L. (1979a) *Biochemistry 18*, 1101–1105.

Bosron, W. F., Li, T.-K., & Vallee, B. L. (1979b) *Biochem. Biophys. Res. Commun. 91*, 1549–1555.

Bosron, W. F., Li, T.-K., & Vallee, B. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5784–5788.

Bosron, W. F., Crabb, D. W., & Li, T.-K. (1982) *Alcohol.: Clin. Exp. Res. 6*, 291.

Bühler, R., & von Wartburg, J.-P. (1982) *FEBS Lett. 144*, 135–139.

Burger, A. R., & Vallee, B. L. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1886.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137.

Cleland, W. W. (1979) *Methods Enzymol. 63A*, 103–138.

Dubied, A., von Wartburg, J.-P., Bohlken, D. P., & Plapp, B. V. (1977) *J. Biol. Chem. 252*, 1464–1470.

Giblett, E. R. (1976) *Cytogenet. Cell Genet. 16*, 65–74.

Harada, S., Agarwal, D. P., & Goedde, H. W. (1978) *Hum. Genet. 40*, 215–220.

Hart, G. E. (1971) *Mol. Gen. Genet. 111*, 61–65.

Lange, L. G., & Vallee, B. L. (1976) *Biochemistry 15*, 4681–4686.

Li, T.-K., Bosron, W. F., Dafeldecker, W. P., Lange, L. G., & Vallee, B. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4378–4381.

Pietruszko, R., Theorell, H., & De Zalenski, C. (1972) *Arch. Biochem. Biophys. 153*, 279–293.

Schenker, T., Teepel, L., & von Wartburg, J.-P. (1971) *Eur. J. Biochem. 24*, 271–279.

Smith, M., Hopkinson, D. A., & Harris, H. (1971) *Ann. Hum. Genet. 34*, 251–271.

Smith, M., Hopkinson, D. A., & Harris, H. (1973) *Ann. Hum. Genet. 36*, 401–414.

Strydom, D. J., & Vallee, B. L. (1982) *Anal. Biochem. 123*, 422–429.

Theorell, H., & Chance, B. (1951) *Acta Chem. Scand. 5*, 1127–1144.

Vallee, B. L., & Bazzone, T. J. (1983) in *Isoenzymes: Current Topics in Biological and Medical Research* (Rattazzi, M. C., Scandalios, J. G., & Whitt, G. S., Eds.) Vol. 8 (in press).

Whitehead, E. P. (1978) *Biochem. J. 171*, 501–504.

Wratten, C. C., & Cleland, W. W. (1963) *Biochemistry 2*, 935–941.

Yoshida, A., Impraim, C. C., & Huang, I.-Y. (1981) *J. Biol. Chem. 256*, 12430–12436.

# Kinetic Properties of Human Liver Alcohol Dehydrogenase: Oxidation of Alcohols by Class I Isoenzymes[†]

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ABSTRACT: Class I isoenzymes of alcohol dehydrogenase (ADH) were isolated by chromatography of human liver homogenates on DEAE-cellulose, 4-[3-[*N*-(6-aminocaproyl)-amino]propyl]pyrazole–Sepharose and CM-cellulose. Eight isoenzymes of different subunit composition ($\alpha\gamma_2$, $\gamma_2\gamma_2$, $\alpha\gamma_1$, $\alpha\beta_1$, $\beta_1\gamma_2$, $\gamma_1\gamma_1$, $\beta_1\gamma_1$, and $\beta_1\beta_1$) were purified, and their activities were measured at pH 10.0 by using ethanol, ethylene glycol, methanol, benzyl alcohol, octanol, cyclohexanol, and 16-hydroxyhexadecanoic acid as substrates. Values of $K_m$ and $k_{cat}$ for all the isoenzymes, except $\beta_1\beta_1$-ADH, were similar for the oxidation of ethanol but varied markedly for other alcohols. The $k_{cat}$ values for $\beta_1\beta_1$-ADH were invariant ($\sim$10 min$^{-1}$) and much lower (5–15-fold) than those for any other class I isoenzyme studied. $K_m$ values for methanol and ethylene glycol were from 5- to 100-fold greater than those for ethanol, depending on the isoenzyme, while those for benzyl alcohol, octanol, and 16-hydroxyhexadecanoic acid were usually 100–1000-fold lower than those for ethanol. The homodimer $\beta_1\beta_1$ had the lowest $k_{cat}/K_m$ value for all alcohols studied except methanol and ethylene glycol; $k_{cat}$ values were relatively constant for all isoenzymes acting on all alcohols, and, hence, specificity was manifested principally in the value of $K_m$. Values of $K_m$ and $k_{cat}/K_m$ revealed for all enzymes examined that the short chain alcohols are the poorest while alcohols with bulky substituents are much better substrates. The experimental values of the kinetic parameters for heterodimers deviate from the calculated average of those of their parent homodimers and, hence, cannot be predicted from the behavior of the latter. Thus, the specificities of both the hetero- and homodimeric isoenzymes of ADH toward a given substrate are characteristics of each. Ethanol proved to be one of the "poorest" substrates examined for all class I isoenzymes which are the predominant forms of the human enzyme. On the basis of kinetic criteria, none of the isoenzymes of class I studied oxidized ethanol in a manner that would indicate an enzymatic preference for that alcohol.

Interest in alcoholism as a metabolic disease has intensified the quest for basic knowledge of the enzyme(s) responsible for the oxidation of ethanol. Blair & Vallee (1966) first demonstrated that the alcohol dehydrogenase (EC 1.1.1.1) (ADH)[1] of human liver is a mixture of isoenzymes, and subsequently more than a dozen isoenzymes of human liver ADH have been recognized (Smith et al., 1973; Bosron et al., 1979a, 1980; Pares & Vallee, 1981). All of the purified ADH isoenzymes of human liver are dimeric proteins composed of

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[1] Abbreviations: ADH, alcohol dehydrogenase; 16-HHA, 16-hydroxyhexadecanoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; CapGapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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